

CRYPTOSPORIDIUM BOVIS N. SP. (APICOMPLEXA: CRYPTOSPORIDIIDAE) IN CATTLE (*BOS TAURUS*)

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ABSTRACT: A new species of *Cryptosporidium*, *C. bovis*, is described. Oocysts of *C. bovis*, previously identified as *Cryptosporidium* genotype Bovine B (GenBank AY120911), are morphologically indistinguishable from those of *C. parvum*. They are excreted fully sporulated and contain 4 sporozoites, but lack sporocysts. Oocysts measure 4.76–5.35 μm (mean = 4.89 μm) \times 4.17–4.76 μm (mean = 4.63 μm), with a length-to-width ratio of 1.06 ($n = 50$). Oocysts were not infectious for neonatal BALB/c mice, but were infectious for 2 calves that were previously infected with *C. parvum*. Oocysts were not infectious for 2 experimentally exposed lambs less than 1 wk of age and were not detected in 42 lambs 2–3 mo of age, but were detected in a 2-wk-old lamb. In an earlier study, 79 of 840 calves on 14 dairy farms in 7 states were found infected with the new species. Most calves were 2–7 mo of age and none exhibited signs of diarrhea. This new species has been found in 10 of 162 calves aged 9 to 11 mo on a beef farm in Maryland. Fragments of the 18S rDNA, HSP-70, and actin genes were amplified by PCR, and purified PCR products were sequenced. Multilocus analysis of the 3 unlinked loci demonstrated the new species to be distinct from *C. parvum* and also demonstrated a lack of recombination, providing further evidence of species status. Based on these biological and molecular data, we consider this highly prevalent *Cryptosporidium* that infects primarily postweaned calves to be a new species and propose the name *Cryptosporidium bovis* n. sp. for this parasite.

In 1910, Tyzzer proposed the name *Cryptosporidium* as a new genus and *Cryptosporidium muris* as the type species found in the gastric mucosa of mice (Tyzzer, 1910). Two years later he proposed the name *Cryptosporidium parvum* for a new species that developed only on the small intestine of mice, and which had smaller oocysts (4.5 μm in greatest diameter) (Tyzzer, 1912). Subsequently, oocysts of *C. parvum* were isolated from infected humans and cattle, DNA was extracted from oocyst isolates, and differences in base pairs within several genes were used to distinguish 2 genotypes. One *C. parvum* genotype was referred to as the anthroponotic “human” genotype, or genotype 1 or genotype H, and the other as the zoonotic “cattle” genotype, or genotype 2 or genotype C (Bonnin et al., 1996; Carraway et al., 1997; Peng et al., 1997; Xiao et al., 1999). Oocysts of *C. parvum* were reported to range in size from 4.5 to 5.4 \times 4.2 to 5.0 μm , with a mean size of 5.0 \times 4.5 μm and a shape index of 1.1 (Upton and Current, 1985), from 4.7 to 6.0 \times 4.4 to 5.0 μm , with a mean size of 5.0 \times 4.7 μm and a shape index of 1.06 (Fayer et al., 2001), and from 5.0 to 5.5 \times 3.7 to 5.0 μm with a mean size of 5.2 \times 4.3 μm and a shape index of 1.20 (Fall et al. 2003). Oocysts overlapping in size and shape to *C. parvum* have now been described from more than 150 animal hosts, but after careful molecular and biological studies, some have been named separate species (Fayer et al. 2000a; Xiao et al., 2004).

Thousands of human cases of cryptosporidiosis have been documented in 95 countries (Casemore et al. 1997; Fayer et al., 2000a; McLaughlin et al. 2000; Pedraza-Diaz et al. 2001; Carey et al., 2004). Genetic analyses of oocyst isolates have shown most human infections were caused by the 2 organisms previously referred to as the “human” and “cattle” genotypes. Isoenzyme analysis, PCR-RFLP, and sequence analysis of many unlinked loci from different geographic locations have dem-

onstrated genetic differences between the “human” and “cattle” genotypes (e.g., Awad-El-Kariem et al., 1995; Bonnin et al., 1996; Carraway et al., 1997; Peng et al., 1997; Spano et al., 1997; Caccio et al., 1999, 2000; Morgan et al., 1999, 2001; Xiao et al., 1999, 2002, 2004; McLaughlin et al., 2000; Pedraza-Diaz et al., 2001; Sulaiman et al., 2002). Biological studies have shown the “cattle” genotype to infect many hosts, whereas the “human” genotype did not readily infect mice or cattle (Casemore et al., 1997; Peng et al., 1997; Widmer et al., 1998, 2000). Based on these molecular and biological differences the “human” genotype was named *Cryptosporidium hominis* (Morgan-Ryan et al., 2002).

A similar pattern has emerged for another genotype of *Cryptosporidium* found in cattle and with oocysts indistinguishable from those of *C. parvum*. *Cryptosporidium* genotype Bovine B was reported from 1 beef calf in Maryland and from a dairy calf in Pennsylvania based on the 18S rRNA gene (Xiao et al., 2002). The sequence for this gene was deposited in GenBank as number AY120911. This genotype was then detected in 79 calves on 14 dairy farms in 7 states in the United States by PCR, and by gene sequencing of the 18S rRNA locus (Santín et al., 2004). In that study, an age biased pattern of infection was observed among 840 calves examined using molecular methods. In preweaned calves (less than 2 mo old) *C. parvum* constituted 85% of the *Cryptosporidium* infections. In postweaned calves (2 to 11 mo old) *C. parvum* constituted only 1% of the infections. In the same preweaned and postweaned calves, genotype Bovine B constituted 9% versus 55% of the *Cryptosporidium* infections, respectively (Santín et al., 2004). Based on these unique molecular and biological characteristics, the present study was conducted to determine whether additional unique attributes were present that indicate *Cryptosporidium* genotype Bovine B is a separate species. As proposed by Xiao et al. (2004) for qualification of species status, morphometric studies were conducted; 3 unlinked loci were sequenced and compared with sequences for the same genes from *C. parvum* and other species and genotypes; and biological uniqueness was determined by feeding oocysts to calves, lambs, and mice to determine the host range of this genotype.

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MATERIALS AND METHODS

Collection of parasites

Feces were collected directly from 971 calves during a survey involving 2 dairy farms in each of 7 states including Florida, Maryland, New York, North Carolina, Pennsylvania, Vermont, and Virginia (Santín et al., 2004), and from 162 beef calves aged 9 to 11 mo from a farm in Maryland. Feces (15 g or more) were placed in plastic specimen cups that were capped and stored on ice for 1–2 days before being examined at the USDA ARS laboratory in Beltsville, Maryland.

Feces were processed as previously described (Fayer et al., 2000b). Briefly, 15 g of feces from each specimen cup were transferred to a 50-ml centrifuge tube containing approximately 35 ml of distilled H₂O, the tube was capped, and then thoroughly mixed using a Vortex-Genie (Scientific Industries, Bohemia, New York). To remove large particles, the fecal suspension was passed through a 45- μ m wire screen into a second 50-ml tube, and the final volume was adjusted to 50 ml with dH₂O. Tubes were centrifuged at 1,800 g for 15 min, the supernatant was discarded, and the pellet was resuspended in 25 ml of dH₂O and mixed well by Vortex-Genie. Twenty-five milliliters of CsCl (1.4 g/L) were added to each tube, the suspension was mixed thoroughly, and the tubes were subjected to a second centrifugation at 300 g for 20 min. Four milliliters of supernatant aspirated from the top of each suspension was transferred to a 15-ml centrifuge tube, and dH₂O was added to reach a final volume of 15 ml. Specimens were centrifuged at 1,800 g for 15 min and washed twice with dH₂O before the final pellet was resuspended in 500 μ l of dH₂O. Portions of the 500- μ l suspension were examined by immunofluorescence microscopy and molecular analysis as described below.

A 100- μ l aliquot of sieved, gradient-cleaned fecal suspension was transferred to a microcentrifuge tube, and washed once with dH₂O. The pellet was resuspended in 50 μ l of premixed Merifluor anti-*Cryptosporidium* reagent (Meridian Diagnostics, Cincinnati, Ohio), and 2 μ l of suspension were transferred to a well (11 mm in diameter) of a 3-well glass microscope slide. The slide was covered with a 24 \times 50 mm coverslip and the entire well area was examined by fluorescence microscopy at \times 400 using a Zeiss Axioskop equipped with epifluorescence and an FITC-Texas Red[®] dual wavelength filter.

DNA extraction, PCR, and sequence analyses

Total DNA was extracted from each of 89 50- μ l suspensions cleaned of fecal debris using a DNeasyTissue Kit (Qiagen, Valencia, California). To increase the quantity of recovered DNA the nucleic acid was eluted in 100 μ l of elution buffer included in the DNeasy Tissue Kit.

Fragments of the 18S rDNA (~830 bp), HSP-70 (~325 bp), and actin (~1,066 bp) genes were amplified by PCR as previously described (Xiao et al., 1999; Morgan et al., 2001; Sulaiman et al., 2002). PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining. PCR products were purified using Exonuclease I/ Shrimp Alkaline Phosphatase (Exo-SAP-IT[®]; USB Corporation, Cleveland, Ohio). Purified products were sequenced in both directions using the same PCR primers in 10- μ l reactions, Big Dye[®] chemistries, and an ABI3100 sequencer analyzer (Applied Biosystems, Foster City, California).

Phylogenetic analyses

The 18S rDNA, HSP-70, and actin sequences were compared with sequences obtained from other *Cryptosporidium* species, and genotypes from GenBank. *Plasmodium falciparum* were used as an out-group for HSP-70 (GenBank M19753), 18S rDNA (GenBank M19172), and actin (GenBank M19146) analyses. Sequences were aligned following the Clustal W algorithm included in the Megalign module (DNASTAR Inc., Madison, Wisconsin). The use of Clustal W determines that once a gap is inserted it can be removed only by editing. Therefore, final alignment adjustments were performed manually to remove artificial gaps. Phylogenetic inference was performed by the Neighbor-Joining method described by Saitou and Nei. (Sequence alignments can be obtained from the authors upon request).

The nucleotide sequences of the 18S rDNA, HSP-70, and actin gene sequences of *C. bovis* isolates have been deposited in GenBank (AY741305, AY741306, and AY741307, respectively). The nucleotide sequence of the HSP-70 gene of *Cryptosporidium* deer genotype has

been deposited in GenBank (AY741308), as has the nucleotide sequence of the actin gene of the *Cryptosporidium* deer-like genotype (GenBank AY741309).

Infectivity for mice, calves, and lambs

Oocysts from 27 bovine fecal specimens from farms in New York and Vermont and individually identified as *Cryptosporidium* genotype Bovine B by PCR and 18S rDNA gene sequencing were cleaned and pooled to conduct transmission studies in mice and 2 calves (nos. 1 and 2). Oocysts from a farm in Maryland were similarly identified by gene sequencing, pooled, and fed to calf no. 3. The 18S rDNA gene was sequenced twice for all isolates included in the pool and after oocysts were pooled.

Three colostrum-deprived Holstein-Friesian male calves were housed separately on wood shavings in pens of 3 m² with cement walls and floor in an isolated cinderblock barn and fed calf milk replacer twice daily. Calf no. 1, less than 1 wk of age, was fed 10⁶ pooled oocysts suspended in water via a nipples bottle. Calf no. 2, 6 wk of age, had previously been experimentally infected with *C. parvum* and had stopped excreting *C. parvum* oocysts. Calf no. 2 was fed 3×10^5 *Cryptosporidium* genotype Bovine B oocysts and, when it stopped excreting oocysts, was fed *C. parvum* oocysts again to test for species-specific immunity. Calf no. 3, 8 wk of age, also previously infected with *C. parvum*, was no longer excreting oocysts when it was fed ~1,500 oocysts of *Cryptosporidium* genotype Bovine B isolated from naturally infected beef calves. Feces were collected from each calf daily for 21–28 consecutive days beginning the day after oocysts were fed.

Pooled oocysts from bovine field isolates were also used to test for infectivity in neonatal BALB/c mice. A total of 2×10^5 oocysts were administered to each mouse (n = 8) by gastric intubation using a 26-ga gavage needle fitted to a micropipette. Mice that received only distilled water served as negative controls (n = 4) and mice that received 2×10^5 oocysts of *C. parvum* (Beltsville strain) obtained from experimentally infected calves served as positive controls (n = 5). All mice were killed by CO₂ asphyxiation and cervical dislocation 5 days after intubation. Tissues from stomach, duodenum, jejunum, ileum, and colon were obtained from each mouse and subjected to DNA extraction and PCR (as described above).

Four colostrum-deprived neonatal Suffolk lambs were purchased from a local farmer; 2 lambs were 3 days of age and 2 lambs were 7 days of age. All were housed separately on wood shavings in pens of 2.5 m² with cement walls and floor in isolated cinderblock buildings and fed lamb milk replacer 3 times daily. One 3-day-old and 1 7-day-old lamb were each fed 2.5×10^3 oocysts of *Cryptosporidium* genotype Bovine. The remaining lambs served as negative controls. Feces from all lambs were collected daily for 21 consecutive days beginning the day after oocysts were fed.

All housing, feeding, and experimental procedures involving calves, lambs, and mice were conducted under protocols approved by the Beltsville Area Animal Care and Use Committee.

Feces were also collected directly from 42 Suffolk, Shropshire, and mixed breed lambs aged 2 to 3 mo on farms in Maryland. Approximately 5 g of feces from each lamb were placed in plastic cups and taken to the USDA laboratory where they were processed on the day of collection.

Oocyst measurements

Oocysts (n = 50) from calf no. 2 were suspended in water, placed on a glass microscope slide with a coverslip, and measured by ocular micrometer at \times 1000 using a Zeiss Axioskop microscope with differential interference contrast microscopy. Oocysts (n = 50) of *C. parvum* from calf no. 1 were measured in the same way by the same person. Photomicrographs were recorded for oocysts observed by differential interference contrast microscopy, and a phototype was deposited in the U.S. National Parasite Collection, Beltsville, Maryland.

RESULTS

Transmission studies

Oocysts identified as *C. bovis* were not infectious for neonatal BALB/c mice, whereas those identified as *C. parvum* were

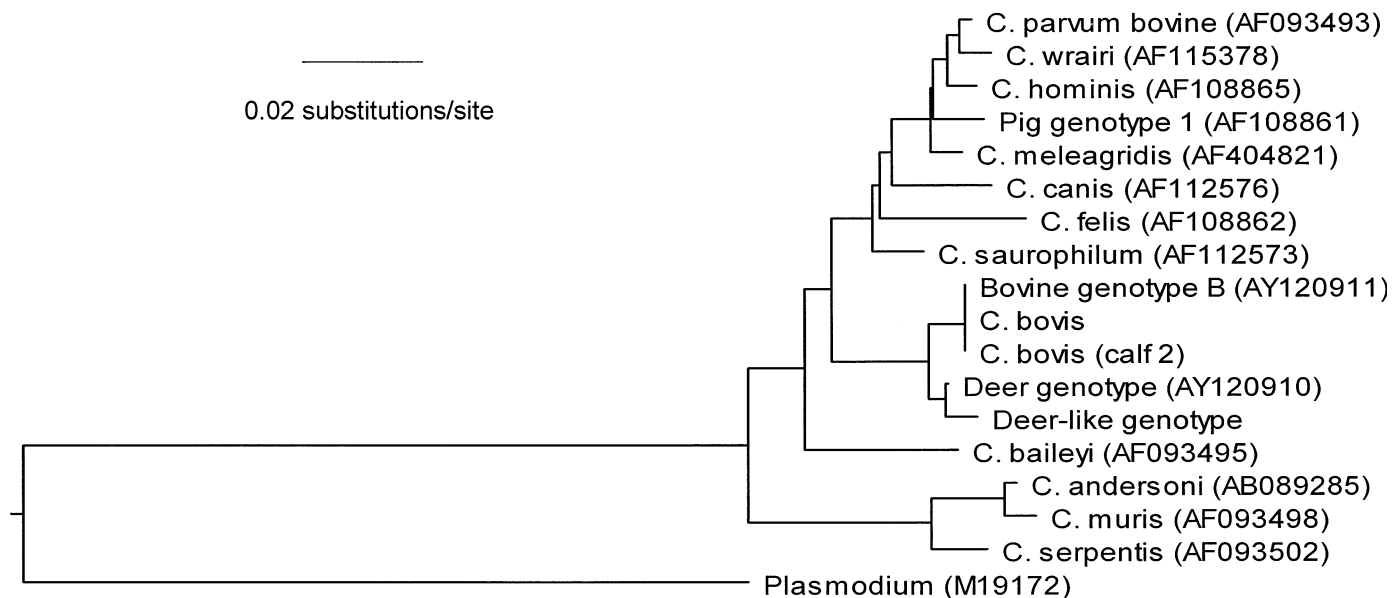


FIGURE 1. Phylogenetic relationships among *Cryptosporidium* species and genotypes inferred by neighbor-joining analysis using a fragment of the 18S rRNA gene.

infectious for all positive control mice as detected by PCR. Neonatal lambs exposed at less than 1 wk of age did not excrete oocysts detectable by either microscopy or PCR. A lamb not experimentally exposed to oocysts of *C. bovis* began to spontaneously excrete oocysts at 16 days of age and continued to excrete oocysts daily for 14 days (confirmed by PCR and sequence data). Of 42 lambs examined on 2 farms in Maryland, 21 were found to excreting oocysts of *Cryptosporidium*, but none was found to excrete *C. bovis*.

Calf no. 1 (less than 1 wk of age) began excreting oocysts 6 days after it was fed a pooled suspension of oocysts from field isolates. Gene sequencing revealed that these oocysts were *C. parvum*, possibly acquired from environmental contamination. However, calf no. 2, an older calf previously infected with *C. parvum*, began excreting oocysts 10 days postinfection. Oocysts were detected in feces by PCR, microscopy (or both) on days 10–19, 21, 23, 25, 26, and 28 postinfection. Genetic analysis

of all these samples revealed that calf no. 2 was excreting *C. bovis* oocysts. When calf no. 2 was later fed *C. parvum* oocysts, it failed to excrete any oocysts during the following 21 days, indicating that immunity to *C. parvum* had not extended to *C. bovis*. Calf no. 3, also previously infected with *C. parvum*, excreted oocysts of *C. bovis* only on day 12 postinfection as determined by gene sequencing.

Gene sequence data

Partial sequences of the 18S rDNA gene were obtained from each of 89 *C. bovis* oocyst isolates primarily from dairy farms in the following states: Vermont (21 isolates), New York (14 isolates), Pennsylvania (11 isolates), Maryland (9 dairy cattle isolates and 10 beef cattle isolates), Virginia (3 isolates), North Carolina (12 isolates), and Florida (9 isolates). These sequences were compared with *Cryptosporidium* sequence data obtained from GenBank. Sequence analysis placed *C. bovis* within a cluster containing *Cryptosporidium* Bovine B genotype (GenBank AY120911), *Cryptosporidium* deer genotype (GenBank AY120910), and *Cryptosporidium* deer-like genotype (GenBank AY587166; Fig. 1).

Partial sequences of the HSP-70 and actin genes were obtained from oocysts pooled from 27 isolates identified as *C. bovis* by PCR followed by sequencing the 18S rRNA gene. These sequences were compared with *Cryptosporidium* sequence information obtained from GenBank. The phylogenetic relationship for the actin gene supported the placement of *C. bovis* in a cluster with *Cryptosporidium* deer (GenBank AY120928) and deer-like (present study) genotypes (Fig. 2). The phylogenetic relationship for the HSP-70 gene supported the placement of *C. bovis* in a cluster with the deer genotype (present study; Fig. 3). Phylogenetic relationships for the 3 genes analyzed (18S rDNA, HSP-70, and actin genes) were

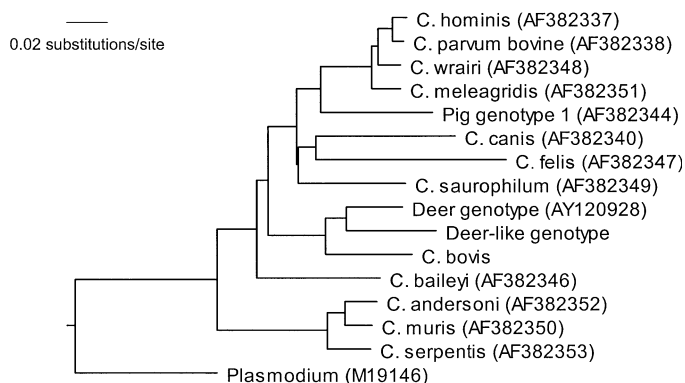


FIGURE 2. Phylogenetic relationships among *Cryptosporidium* species and genotypes inferred by neighbor-joining analysis using a fragment of the actin gene.

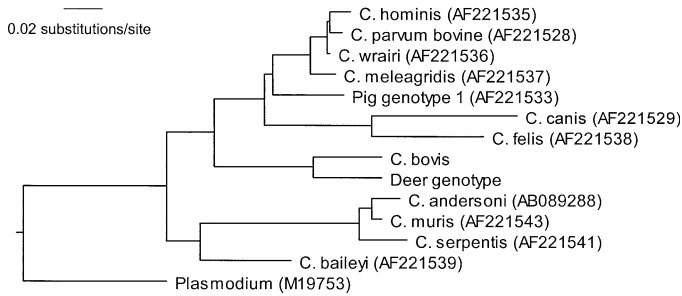


FIGURE 3. Phylogenetic relationships among *Cryptosporidium* species and genotypes inferred by neighbor-joining analysis using a fragment of the HSP-70 gene.

consistent, with the *C. bovis* forming a distinct cluster with the deer and deer-like genotypes.

DESCRIPTION

Cryptosporidium bovis n. sp. (Figs. 4–6)

Diagnosis: Oocysts are shed in feces fully sporulated. Oocysts ($n = 50$) measure $4.76\text{--}5.35 \times 4.17\text{--}4.76 \mu\text{m}$, with a mean size of $4.89 \times 4.63 \mu\text{m}$, and a shape index of 1.06 (Figs. 4–6). Four sporozoites are present in each oocyst. The prepatent period is 10 days, the patent period is 18 days. Oocysts contained 4 sporozoites but had no sporocysts. Endogenous stages are unknown.

Type host: Cattle, *Bos taurus*.

Other hosts: Sheep, *Ovis aries* (minor).

Type locality: United States.

Other localities: Australia (sheep).

Material deposited: A phototype of sporulated oocysts and DNA is deposited in the United States National Parasite Collection (USNPC), Beltsville, Maryland as USNPC 94840.

Etymology: This species is named *Cryptosporidium bovis* to reflect its major host type.

DISCUSSION

Oocysts of *C. bovis* were found to be morphologically indistinguishable from those of *C. parvum*. Fifty oocysts of *C. parvum* processed and measured under the same conditions by the same person in the present study measured $4.76\text{--}5.95 \times 4.76\text{--}5.35 \mu\text{m}$, with a mean size of $5.47 \times 5.15 \mu\text{m}$. Oocyst char-

acteristics other than size and shape, such as color, appearance of contents, and thickness of oocyst wall, were indistinguishable between the 2 species. The mean sizes reported for *C. parvum* oocysts in other studies were $5.0 \times 4.5 \mu\text{m}$, $5.0 \times 4.7 \mu\text{m}$, and $5.2 \times 4.3 \mu\text{m}$ (Upton and Current, 1985; Fayer et al., 2001; Fall et al., 2003), all very close to the mean size of $4.89 \times 4.63 \mu\text{m}$ found for oocysts of *C. bovis* in the present study. In addition to similarity in size, both species have the same shape index of 1.06.

Genetically confirmed *C. bovis* infection, previously named Bovine B genotype, has been found in dairy and beef cattle (Xiao et al., 2002; Santín et al. 2004) and in mature sheep in Western Australia (Ryan et al., in press). Cattle infected with *C. bovis* were geographically widespread, covering an overall distance of more than 2,100 km on farms with a wide range of climatic, soil, and management conditions along the east coast of the United States (Santín et al., 2004).

Two calves that had been experimentally infected with *C. parvum* were susceptible to experimental infection with *C. bovis*, indicating that immunity to 1 species of *Cryptosporidium* did not impart immunity to the other species. Another biologic difference between *C. parvum* and *C. bovis* is an age-related bias; 85% versus 9% of *Cryptosporidium*-positive dairy calves 2 mo of age and younger were infected with *C. parvum* versus *C. bovis*, whereas 55% versus 1% of dairy calves 2 to 11 mo of age were infected with *C. bovis* versus *C. parvum* (Santín et al., 2004). In that study, another distinct biological feature was reported. None of the calves found infected with *C. bovis* had any signs of disease, whereas calves infected with *C. parvum* often have been reported as diarrheic (de Graaf et al., 1999).

Oocysts of *C. bovis* failed to infect any of 8 neonatal mice and 2 neonatal lambs in the present study, although oocysts from the same pool were infectious for a calf. In contrast, oocysts of *C. parvum* readily infected neonatal BALB/c mice in the present study and are known to be infectious for mice and lambs from earlier studies (McLauchlin et al., 2000). Mice and lambs were neonates and might not have been susceptible to infection with *C. bovis* if this species has a host age-related requirement that crosses host species lines.

Phylogenetic analyses confirm the validity of *C. bovis* at 3 independent loci. The 3 loci data sets provide strong support for the genetic distinctiveness of *C. bovis* from *C. parvum* and the clustering of *C. bovis* with the *Cryptosporidium* deer genotype and the *Cryptosporidium* deer-like genotype. This clustering of related species/genotypes of *Cryptosporidium* harbored by genetically related hosts has been recognized by an

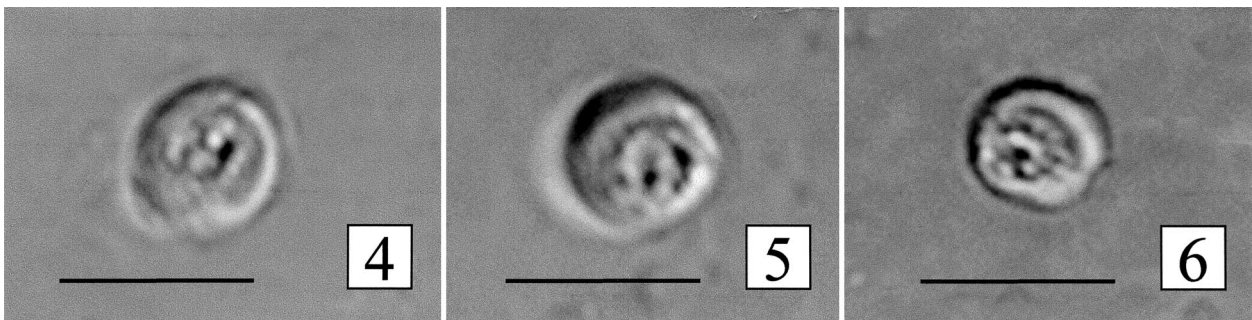


FIGURE 4–6. Differential interference contrast photomicrographs of oocysts of *Cryptosporidium bovis* n. sp. Bar = $5 \mu\text{m}$.

earlier study (Xiao et al., 2002). Phylogenetic analyses of the 18S rDNA locus revealed that *C. bovis* shared only 95.1% similarity with *C. parvum* and 94.5% with *C. hominis*. This is much less than the similarities among already recognized *Cryptosporidium* species such *C. meleagridis*, *C. parvum*, and *C. hominis* (98.5 to 99.1%), and between *C. parvum* and *C. hominis* (99.1%).

Genetic differences from 3 independent loci combined with biological differences relating to natural and cross-transmission host specificity, the age of the host, lack of cross-species immunity, and lack of pathogenicity indicate that *C. bovis* is a distinct species. The correct identification of *Cryptosporidium* species in clinical and epidemiological specimens has important and far reaching veterinary and public health implications. Cattle of all ages have been found infected with *Cryptosporidium*. Some animals suffer severe illness and others appear healthy. The diagnosis, often made by microscopic identification of the oocyst stage but infrequently confirmed by molecular methods, has led to the widespread misconception that cattle of all ages are major sources of the zoonotic species *C. parvum* that is pathogenic for humans, cattle, and other animals. Identification of *C. bovis* as the primary species of *Cryptosporidium* found in postweaned cattle, a species not found to infect humans or to cause illness in livestock, provides clarification to the complicated epidemiologic paradigm associated with the genus *Cryptosporidium*.

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